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REGULAR PAPER

Mutation in the cysteine bridge domain of the γ-subunit affects light regulation of the ATP synthase but not photosynthesis or growth in Arabidopsis

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Abstract The chloroplast ATP synthase synthesizes ATP from ADP and free phosphate coupled by the electrochemical potential across the thylakoid membrane in the light. The light-dependent regulation of ATP synthase activity is carried out in part through redox modulation of a cysteine disulfide bridge in CF_1 γ-subunit. In order to investigate the function of the redox regulatory domain and the physiological significance of redox modulation for higher plants, we designed four mutations in the redox regulatory domain of the γ-subunit to create functional mimics of the permanently reduced form of the γ-subunit. While the inability to reduce the regulatory disulfide results in lower photosynthesis and growth, unexpectedly, the results reported here show that inability to reoxidize the dithiol may not be of any direct detriment to plant photosynthetic performance or growth.

Keywords Chloroplast ATP synthase · Disulfide bond · Redox modulation · Thioredoxin

Abbreviations

CF_1	Coupling factor 1
DCCD	*N*, *N*′-Dicyclohexylcarbodiimide
DTT	Dithiothreitol
RT-PCR	Reverse transcript polymerase chain reaction

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Introduction

The proton-translocating F_0F_1-ATP synthase is a ubiquitous, multi-subunit enzyme present in the plasma membrane of eubacteria, the inner mitochondrial membrane, and the photosynthetic membranes of higher plants, algae, and cyanobacteria. The active form of ATP synthase can catalyze both the formation and hydrolysis of ATP. Thus, control over ATP synthetic and hydrolytic activity is of vital importance for energy transduction. While the activities of all ATP synthases are intricately regulated by several mechanisms, redox regulation (also called thiol modulation) is a unique feature of the chloroplast ATP synthase from algae and higher plants (reviewed in Ort and Oxborough 1992; Richter 2004). The CF1 γ-subunit contains an intra-peptide disulfide bond between two cysteines located in an extra domain of the chloroplast subunit not present in mitochondrial or bacterial γ-subunit sequences (Miki et al. 1988). This cysteine disulfide is the target of thioredoxin in the physiological reduction pathway (Shahak 1982; Ketcham et al. 1984). The reduction of the disulfide bond is thought to cause a conformational change within the enzyme complex thereby decreasing the magnitude of the proton motive force ($\Delta\mu_H^+$) required to maintain a catalytic activity (Nalin and McCarty 1984) and increasing photophosphorylation at limiting $\Delta\mu_H^+$ (Hangarter and Ort 1986; Hangarter et al. 1987).

In the case of the γ-subunit of Arabidopsis, the two regulatory cysteines are located at positions 199 and 205 of the amino acid sequence through which thiol modulation occurs diurnally under normal day/night cycles (Kramer

et al. 1990). In vivo, these cysteines can be oxidized to a disulfide bond in the dark and re-reduced to thiols by the small stromal protein thioredoxin in the light (Ketcham et al. 1984). This thioredoxin-mediated oxidation reaction has a redox midpoint potential ($E_{m,7.9}$) of −335 mV (Hutchison et al. 2000; Wu et al. 2007). Reduction can be achieved by dithiothreitol (DTT) treatment in vitro but the interaction is much less efficient than for thioredoxin (Arana and Vallejos 1982; Dann and McCarty 1992). Site-directed mutagenesis experiments both in vivo with *Chlamydomonas reinhardtii* and in vitro with spinach showed that a serine or alanine substitution of the cysteine in the regulatory bridge domain results in the loss of redox modulation of ATP synthase activity (Ross et al. 1995; Samra et al. 2006). Insertion of a chloroplast-like regulatory domain into the cyanobacterium ATP synthase enabled the enzyme to respond to redox regulation (Werner-Grune et al. 1994). A single amino acid substitution (E244K) in the vicinity of the redox modulation domain causes the γ-subunit to be much more difficult to reduce due to a 39 mV decrease in the $E_{m,7.9}$ of the regulatory dithiol/disulfide couple (Wu et al. 2007). Deletion of residues Glu210–Glu212 following the two cysteines causes the ATPase complex to be inactivated rather than activated by reduction of the disulfide bond. In contrast, deletion of Glu212 to Ile230 conferred high ATPase activity independent of DTT treatment (Konno et al. 2000).

Significant progress has been made in understanding the structure, function, and regulatory mechanism of ATP synthase in recent years. However, our knowledge about the physiological significance of thiol modulation of the γ-subunit is still ambiguous. It appears that the oxidation is a redundant process because the deactivation of the chloroplast ATP synthase is much faster than the oxidation step following a light-to-dark transition (Noctor and Mills 1988; Ortiz-Lopez et al. 1991). When the two cysteines in the *Chlamydomonas* enzyme were replaced by serines to prevent any possibility of formation of disulfide bond, the mutant still grew as fast as wild type under 8-h light/16-h dark period indicating that the enzyme was still converting to a deactivated state in the dark (Ross et al. 1995). In addition, *Chlamydomonas* had no obvious disadvantage to wild type due to the removal of the regulatory cysteines (Ross et al. 1995). However, the opposite circumstance, the inability to reduce the disulfide bridge, is detrimental to photosynthesis and growth. Our recent study on the *cfq* mutant of Arabidopsis, in which the ATP synthase is energetically difficult to reduce, showed that inability to reduce the γ-subunit disulfide bond resulted in diminished photosynthesis and growth (Wu et al. 2007). In order to further investigate the significance of the regulatory domain and the physiological significance of redox modulation, we designed four site-directed mutants in Arabidopsis (*Arabidopsis thaliana*) of the γ-subunit gene—C199S, C205S, C199/205S, and Δ197–205 (i.e., full deletion of regulatory domain)—to study the physiological significance and consequences of γ-subunit oxidation on photosynthesis and growth.

Materials and methods

Plant materials and growth conditions

Plants were grown in soil at 20°C, 70–80% relative humidity under 12/12 h light/dark photoperiod with photon flux density (*PFD*) about 100–200 μmol m^{-2} s^{-1}.

CF_1 γ-subunit constructs

Using QuickChange Site-Directed Mutagenesis kit (Stratagene), the cysteine codons at positions 199 and/or 205 were substituted with serine codons using PBI-121 binary vector containing the atpC1 cDNA as template. Those three different mutations were designated as C199S, C205S, and C199/205S (Fig. 1). The primer pairs used for the mutant γ-subunits were as follows: 5′-AAAGGAGAGATC*TCT*GACATTAATGG-AACC-3′ and 5′-TCCATTAATGTC*AGA*GAT-CTCTCCTTTAGG-3′ for C199S, 5′-ATTAATGG-AACC*TCC*GTGGATGCTGCGGAA-3′ and 5′-CGCAGCATCCAC*GGA*GGTTCCATTAATGTC-3′ for C205S, 5′-GAGATC*TCT*G-ACATTAATGGAACC*TCC*GTGGATGCTGCGGA-3′ and 5′-ATCCAC*GGA*GGTTCCATT-AATGTC*AGA*GATCTCTCCTTTAGG-3′ for C199/205S. The serine codons that replaced cysteine codons are underlined in the primer sequences. The fourth mutation (Δ197–205) was generated by deleting nine amino acid residues within the regulatory domain that included the two redox active cysteine residues (Fig. 1), using the oligonucleotide 5′-TCACCTAAAGGAGTGGATG-CTGCGGAAGATGAGTTT-3′ and 5′-CGCAGCATCCACTCCTTTAGGTGATAAAGGCA-GTAG-3′. A restriction pattern was introduced into each mutant construct. For C199S, C199/205S, and Δ197–205, their native Tsp45I restriction site was eliminated. For C205S, a BsaJI restriction site was added. The products were confirmed by sequencing. These mutations were introduced into plants using *Agrobacterium*-based transformation of wild-type (Col) plants (Clough and Bent 1998). Plants carrying the transgenes were isolated through kanamycin selection. The homozygous lines for the transgenes were obtained in the T_3 generation.

DNA gel blot analysis

To confirm the presence of the transgenes, the genomic DNA was extracted from 3-week-old plants according to

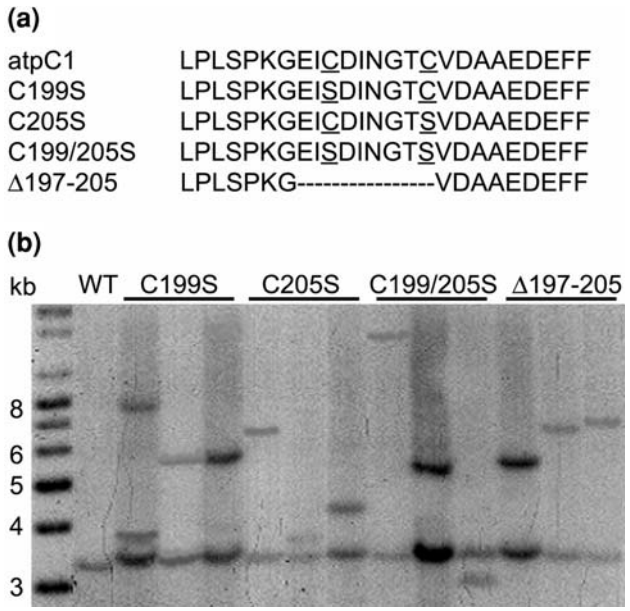

Fig. 1 Site-directed mutation constructs in the redox regulatory domain of the chloroplast ATP synthase and DNA blotting analysis in transgenic plants. (**a**) WT redox regulatory domain and four mutation constructs are shown. Cysteines and substituting serines are underlined. (**b**) DNA gel blot analysis of atpC1 in the wild-type and the transgenic plants. DNA gel blot analysis used 8 μg of EcoRV digested genomic DNA which was loaded in each lane and probed with a randomly labeled BamHI-MboI fragment from the λC31 corresponding to the transit sequence of atpC1 (Inohara et al. 1991). Molecular mass markers are indicated at the left. Three independent transgenic lines from each construct were analyzed. In order to confirm the presence of the additional restriction digest bands, the lanes were loaded with differing amounts of sample as indicated by the different background levels of the various lanes

the procedure described elsewhere (Edwards et al. 1991). Approximately 8 μg DNA was digested overnight with EcoRV, separated on 0.7% (w/v) agarose gels, and transferred onto Hybond-N nylon membrane (Amersham Pharmacia Biotech UK Limited). A BamHI-MboI fragment from the λC31 corresponding to transit sequence was used as a probe to confirm transformation (Inohara et al. 1991). α-[^{32}P]dCTP-labeled probe was prepared using the Megaprime DNA Labelling System (Amersham Pharmacia Biotech UK Limited). Blots were hybridized at 60°C in hybridization solution overnight. The blots were repeatedly washed for 15 min in 0.2× SSC and 0.1% (w/v) SDS and thereafter exposed to X-ray film (Kodak X-Omat AR) for 1 day.

Reverse transcription PCR and restriction analysis

Total RNA was isolated from 4-week-old plants using Trizol reagent (Gibco/BRL). A total of 0.2 μg RNA was treated with 1 U RQ1 DNase (Promega) for 30 min at 37°C. After inactivation of DNase by heating at 70°C for 10 min, the RNA was used as the template for cDNA synthesis using the primers 5′-TCTGTTTCACCACTCCA AGCGTCT-3′ and 5′-TCAAAGAGGGTTCAAAACAAA TCAAAC-3′ to amplify a 1,010 bp fragment containing the mutation sites in the γ-subunit. The RT-PCR products from C199S, C199/205S, and Δ197–205 were digested with Tsp45I, and the RT-PCR products from C205S were digested with BsaJI. The resulting products were separated on 1% (w/v) agarose gel. The intensities of bands were quantified by using Photoshop 6.0 (Adobe Systems Incorporated).

Gel electrophoresis and western blotting

The chloroplast thylakoid membranes were isolated from the 4-week-old leaves according to the protocol published elsewhere (Kee et al. 1986; Whitmarsh and Ort 1984). Antiserum raised against the spinach chloroplast ATP synthase β-subunits was used for detection of CF_1 β-subunits. Western blotting was performed as described elsewhere (Wu et al. 2007). Band intensities were analyzed using Photoshop 6.0 (Adobe Systems Incorporated).

Measurement of ΔA_{518} relaxation kinetics

Plants about 45 days old were initially dark adapted for 12 h to fully oxidize the ATP synthase CF γ-subunit. A kinetic spectrophotometer was used to monitor the absorbance change at 518 nm and the relaxation time constants were calculated as described (Ortiz-Lopez et al. 1990; Wu et al. 2007). For preillumination treatment, dark-adapted leaves were illuminated with 65 μmol m^{-2} s^{-1} red light for 7 s, then dark readapted for times as described in the text.

For DCCD treatment, detached leaves were submerged in a solution of 5 mM DCCD, 2% methanol, and 1% (v/v) Tween-20 for 1 h in the dark. For control, leaves were treated for the same period with 2% methanol and 1% (v/v) Tween-20.

For DTT treatment, detached leaves were incubated in a solution containing 10 mM DTT and 1% (v/v) Tween-20 for 20 min in the dark. For control, leaves were treated with 1% (v/v) Tween-20.

Measurement of ATPase hydrolysis activity

The chloroplast thylakoid membranes were prepared as described above. ATPase hydrolysis activities were analyzed according to the previously described method (Wu et al. 2007).

Gas exchange measurement

Leaf gas exchange measurements were conducted using an open gas exchange system (LI-6400; LI-COR, Inc., Lincoln,

NE). Prior to measurements, plants were dark adapted for 1 h. Gas exchange measurements were conducted on single rosette leaves. The chamber was set at 21°C, 60–70% relative humidity and [CO_2] was set to 370 μmol mol^{-1}. Each experimental treatment was performed with five replications. Data were analyzed with SAS (version 8.02; SAS Institute, Cary, NC).

Growth rate analysis

For growth rate analysis, plants were grown at 50, 100, or 150 μmol m^{-2} s^{-1} white light, 12/12 h light/dark photoperiod, 70–80% humidity. After 30 days, above-ground shoot fresh weights were measured.

Results

Mutant γ-subunit design

Figure 1a illustrates the motifs in the γ-subunit of chloroplast ATP synthase contributing to thiol modulation. This region contains two strongly conserved cysteine residues, Cys199 and Cys205, not present in *E. coli* ATPase, mitochondria ATPase, or cyanobacterium ATP synthase. To further study the significance of thiol modulation, each cysteine individually as well as in combination was replaced by serine through site-directed mutagenesis. A deletion mutation of the nine amino acid cassette containing the regulatory cysteines (Δ197–205) was also designed. Because the codon for cysteine was changed to the codon for serine or the nine amino acid sequence was deleted, disulfide formation between the native cysteines was abolished. Therefore, the mutant γ-subunit gene products can be considered to be mimics of a "permanently reduced" configuration. The constructs were positioned downstream of the CaMV 35S promoter and transformed into wild-type Arabidopsis plants (Col). The presence of the transgene constructs was confirmed by DNA gel blot analysis (Fig. 1b). Due to the low homology in the coding regions for the transit sequences between the atpC1 and atpC2 genes, the transit sequences could be used as specific probes to distinguish between the two genes. Probing the genomic DNA of the wild type with the BamHI-MboI fragment from the λC31 corresponding to transit sequence resulted in a single EcoRV band about 3.4 kb as expected based on the location of atpC1 in Arabidopsis genome. In T_3 transgenic lines, a second band of various sizes was observed, demonstrating that all of the constructs were successfully integrated into the genome of the tested transformants.

Transgene expression in the transgenic plants

In order to evaluate the expression and quantify the level of expression of the transgenes, we intentionally introduced new restriction patterns into the mutant gene constructs, as described in Materials and Methods. RT-PCR was performed to amplify a 1,010 bp fragment containing the mutation site. Tsp45 I was used for restriction analysis of the RT-PCR products of C199S, C199/205S, and Δ197–205, and Bsa JI was used for the C205S product. For the wild-type plant, the 1,010 bp RT-PCR product had two Tsp45 I sites and digestion resulted in 262 bp, 369 bp, and 380 bp fragments, whereas for C199S and C199/205S, their products lost a Tsp45I site at the mutation site and resulted in only 262 bp and 749 bp fragments (Fig. 2a). Similar products were generated in Δ197–205 where a 722 bp product replaced the 749 bp fragment. In the transgenic plants, the 749 bp or 722 bp bands are much brighter than 369 bp and 380 bp bands indicating that the transgenes were more strongly expressed than the native gene. In C205S, we introduced a BsaJI site that resulted in two intense bands at 378 bp and 632 bp. The 1,010 bp band was more intense than expected due to incomplete

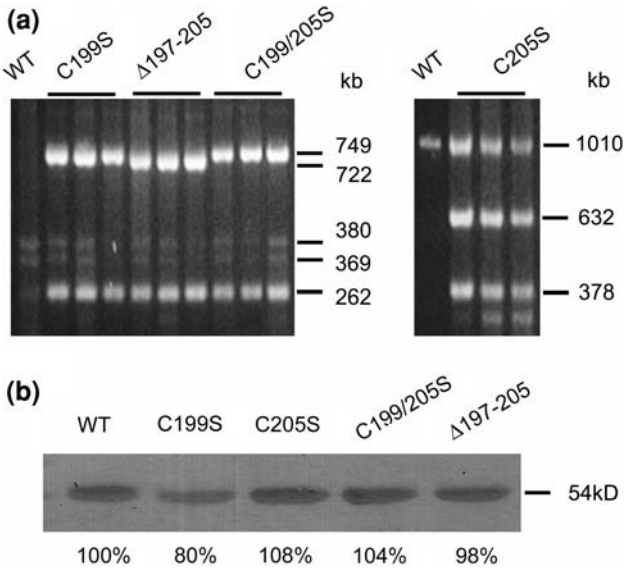

Fig. 2 Transgenes were overexpressed in the transgenic plants and the overexpression does not alter the overall ATP synthase content in the thylakoid membrane. (**a**) RT-PCR and restriction analysis of transgenic plants. The mRNA was isolated from transgenic plants and the fragments containing regulatory site were amplified by RT-PCR. Thereafter, the products from C199S, C199/205S, and Δ197–205 were digested with Tsp45I, and the C205S products were digested with BsaJI and resolved in DNA gel. (**b**) Immunoblot analysis of β-subunit. The CF_1 of ATP synthase was isolated from the thylakoid membrane containing 55 nmol chlorophyll and separated by SDS-PAGE and transferred to nylon membrane. Immunoblotting was performed using anti-β-subunit antiserum. The relative intensity of each band is given as percentage of control

digestion (Fig. 2a). The results showed that the transgenes were stably overexpressed, and that the expression level was about 10 times higher than that of endogenous γ-subunit gene.

Immunoblot analysis

It is possible that overexpression of *atpC1* may have increased the content of the ATP synthase in thylakoid membrane. In order to investigate this possibility, immunoblotting was performed to determine the amount of ATP synthase β-subunit. Since each ATP synthase contains three β-subunits, the total chloroplast ATP synthase content could be estimated on this basis. Figure 2b shows that there were no significant differences in the β-subunit content among the wild type and C205S, C199/205S, and Δ197–205 transgenic plants, indicating that overexpression of the constructs had no significant influence on the overall titer of the ATP synthase.

ΔA_{518} relaxation kinetics

In vivo ATP synthase activity was monitored using the flash-induced absorbance change at 518 nm (Wu et al. 2007). In wild-type dark-adapted leaves, a single saturating flash was insufficient to activate the ATP synthase and thus relaxation of Δ518 was slow (time constant of ~450 ms; Fig. 3a). Preillumination of wild-type leaves for 7 s with 65 μmol m^{-2} s^{-1} red light resulted in the reduction of the γ-subunit and thereafter a single saturating flash could activate the ATP synthase accelerating Δ518 relaxation (Fig. 3a; Wu et al. 2007). In contrast to the wild type, the high flash-induced activity of ATP synthase (i.e., rapid relaxation of Δ518) in three of the transgenic mutants is independent of preillumination (Fig. 3a). The flash-induced Δ518 relaxation kinetics of C199S, C205S, and C199/205S transgenic lines were substantially more rapid in dark-adapted plants than for control showing that the predicted "reduced" behavior accompanied incorporation of the transgenic forms of the γ-subunit into the ATP synthase of the transgenic mutant plants. Similar results were obtained in two other independent lines (data not shown). It is likely that the strong expression of transgenes helped maximize the incorporation of transgene products, γ-subunits, into the assembled ATP synthase. A small amount of wild-type γ-subunits was evidently integrated into the ATP synthase because preillumination increased the activity of ATP synthase to a small extent (Fig. 3a). The ATP synthase behavior in Δ197–205 mutant was very similar to that of the wild type, perhaps because this construct did not integrate into the enzyme due to an incompatible conformation due to

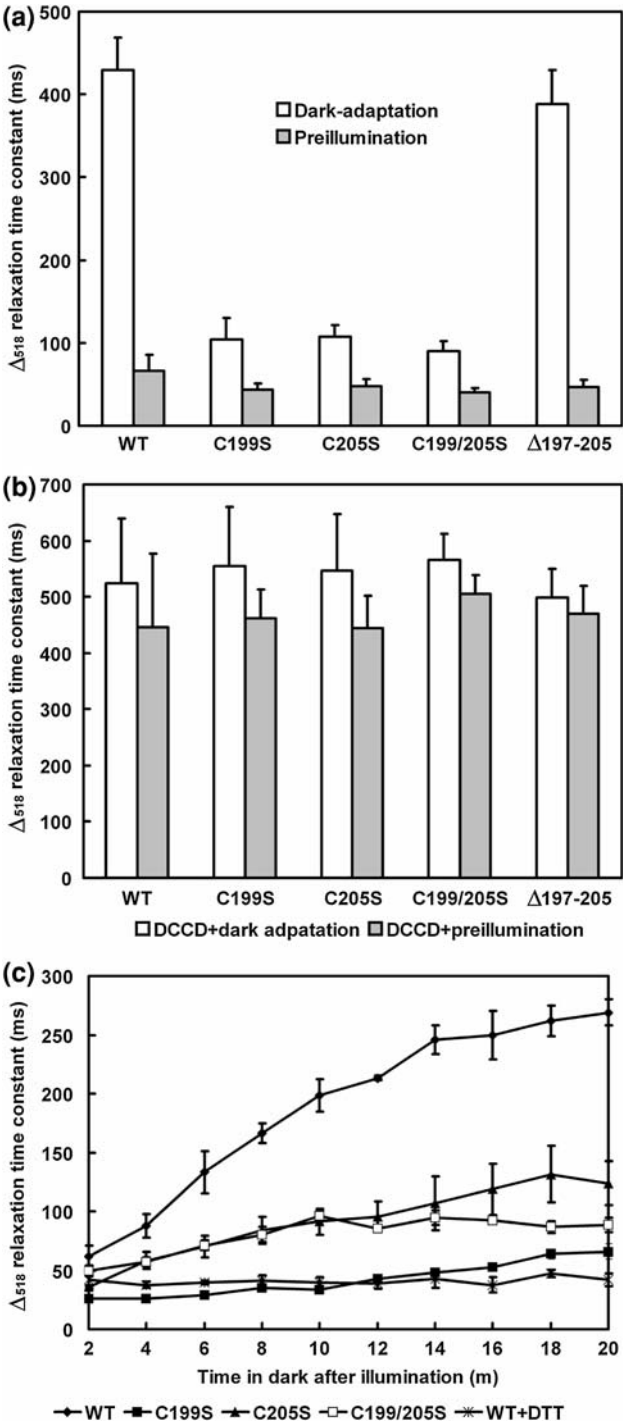

Fig. 3 ΔA_{518} relaxation kinetic analysis of ATP synthase activity in the wild-type and transgenic lines. (**a**) The chloroplast ATP synthase activity represented by ΔA_{518} relaxation time constant before and after exposure to light. (**b**) ΔA_{518} relaxation time constant with DCCD treatment. (**c**) Deactivation of chloroplast ATP synthase in the dark. The leaves were dark adapted for 12 h, then the dark-adapted leaves were exposed to 65 μmol m^{-2} s^{-1} red light for 7 s to fully activate the enzyme. Thereafter, the leaves were dark-adapted again and ΔA_{518} relaxation kinetics was measured at times indicated. The mean values from five plants are shown. Error bars represent standard errors

the nine amino acid deletion. For this reason, the deletion mutant was not further investigated in this study. In order to rule out the possibility that the mutant γ-subunit in ATP synthase changed the properties of the thylakoid membrane to cause the membrane to be more leaky to protons, DCCD, which inhibits ATP synthesis by blocking proton flow through the ATP synthase CF_0, was used to treat the leaves for 1 h. The ΔA_{518} relaxation time constants were significantly increased in the DCCD-treated transgenic plants (Fig. 3b), indicating that the integrity of the thylakoid membrane was unchanged in the mutants.

Deactivation of chloroplast ATP synthase in the dark

The impact of the "permanently reduced" configuration of the mutated γ-subunits on the process of deactivation of ATP synthase was investigated. The ATP synthase for the wild type took more than 20 min to fully deactivate in the dark after activation by 7 s of 65 μmol m^{-2} s^{-1} red light illumination (Fig. 3c). However, the deactivation of ATP synthase was extremely slow and incomplete in C199S, C205S, and C199/205S plants showing that the deactivation of the enzyme in these mutants was comparable to that of DTT-treated wild-type leaves.

Activation of CF_1 ATPase activity

The isolated chloroplast ATP synthase normally has low ATPase activity. This latent enzyme can be activated by thioredoxin in the presence of DTT (Stumpp et al. 1999). A study of ATPase activation by thiol modulation in spinach showed that while chloroplast Trx-f is most efficient, both chloroplast Trx-m and *E. coli* thioredoxin were capable of activating the ATPase (Schwarz et al. 1997). We investigated the effect of the mutations on the thiol modulation of ATP synthase via *E. coli* thioredoxin in the presence of DTT. ATP hydrolysis activity (i.e., ATPase activity) of isolated thylakoid membranes from wild-type leaves was low (Fig. 4a). However, the ATPase activities in the three cysteine-to-serine substitution mutants were about three times higher than that of wild type, similar to the level of the wild type after DTT treatment.

In vivo or in isolated chloroplasts, low light can activate the chloroplast ATP synthase effectively and quickly (Kramer et al. 1990). Illumination increased the wild-type ATPase activity substantially saturating at ~100 μmol m^{-2} s^{-1} for a 2 min exposure. In contrast, illumination only slightly enhanced the already high ATPase activity in the mutants, consistent with the result observed with DTT treatment (Fig. 4b).

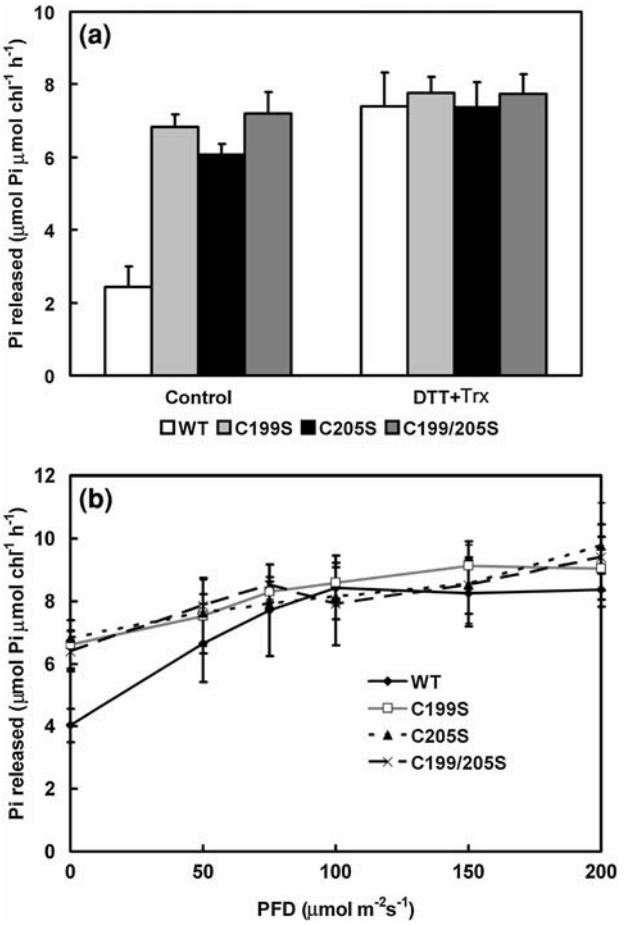

Fig. 4 (**a**) Activation of ATPase by thioredoxin in the presence of DTT. The thylakoid membrane was incubated with or without 2 μM *E. coli* thioredoxin and 2 mM DTT for 15 min at 30°C. ATPase activity was then measured. (**b**) Induction of ATP synthase activity by illumination with light for 2 min in thylakoid membrane. Each data point represents the average of four individual samples ±SE

Photosynthesis activity and plant growth

We investigated photosynthesis by analyzing CO_2 assimilation rates in both the serine-for-cysteine substitution transformants and wild-type plants at different light level intensities (Fig. 5a). The CO_2 assimilation rates at three light intensities showed no significant differences between the wild type and the transformants. Since the mutant γ-subunits delayed deactivation of the enzyme, and the ATPase activity in the transformants was not fully inhibited in the dark, there could be substantial effects on overall plant growth without large changes in rate of net photosynthesis in the light. To address this question, we measured shoot fresh weight but found no evidence of a difference in shoot fresh weight between the wild type and the transformants (Fig. 5b).

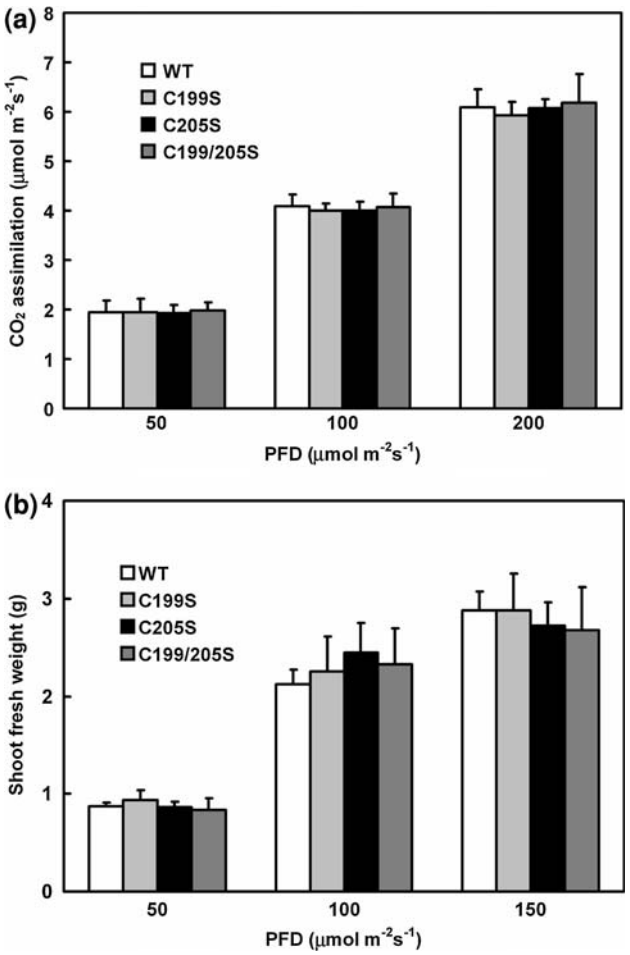

Fig. 5 CO_2 assimilation rate and shoot growth rate. (**a**) No difference in CO_2 assimilation rates. Plants were grown at 20°C, 70–80% relative humidity under 10/14 h light/dark photoperiod with photon flux density (PFD) of 100–200 μmol m^{-2} s^{-1} until bolting. Each data point represents the average of five or six individual plants ±SE. (**b**) No difference in the shoot growth. Plants were grown at 50, 100, and 150 μmol m^{-2} s^{-1} white light. After 30 days, shoot fresh weights were measured. Each data point represents the fresh weight of five plants with average of three measurements ±SE

Discussion

Although the redox domain in the γ-subunit is unique to green algae and plants, it appears that it is structurally and functionally compatible with the ATP synthase of organisms normally lacking it. In vivo, Konno et al. (2000) succeeded in reconstituting $\alpha_3\beta_3\gamma$ complex with enzymatic activity from the recombinant α- and β-subunits from *Bacillus* PS3 and recombinant spinach γ-subunit with a mutation in the conservative segment following the redox regulatory cysteine residues. In vitro, Werner-Grune et al. (1994) introduced a γ-subunit containing a regulatory segment responsible for thiol modulation into F_0F_1-ATPase of the cyanobacterium *Synechocystis* 6803, successfully conferring redox modulation of activity. Thus, it is clear that redox modulation of ATP synthase activity can be introduced even when it is naturally absent. It has also been demonstrated that redox modulation of ATP synthase activity can be removed from organisms where it is normally present. Ross et al. (1995) replaced the two cysteines by serines in the γ-subunit of F_0F_1-ATPase from the green alga *Chlamydomonas reinhardtii* which abolished redox modulation of activity. However, all of this previous work focused on the effect of redox regulation on the ATP synthase enzyme itself, while the important information about the significance of the redox domain on photosynthetic performance was not investigated.

Recently, we reported that a point mutation in the vicinity of the redox regulatory domain prevented redox modulation in the Arabidopsis ATP synthase by decreasing the redox midpoint potential of the regulatory disulfide out of the range of thioredoxin. Loss of redox modulation caused lower ATP synthesis capacity, which in turn restricted overall rates of leaf photosynthesis under low light (Wu et al. 2007). This work provided in situ validation that thioredoxin-dependent reduction of the γ-subunit regulatory disulfide modulates the proton electrochemical potential energy requirement for activation of the chloroplast ATP synthase and that the activation state of the ATP synthase can limit leaf level photosynthesis.

In the work reported here we sought to investigate consequences of the reciprocal circumstance in which the thylakoid ATP synthase is locked into the mimic of a permanently reduced conformation. For this purpose, we designed four mutant constructs of the γ-subunit redox domain, C199S, C205S, C199/205S, and Δ197–205, and transformed wild-type plants with these constructs under the control of strong 35S promoter. Overexpression of the four constructs neither changed the ATP synthase content (Fig. 2b) nor affected the integrity of the thylakoid membrane (Fig. 3b). As expected, the mutations of C199S, C205S, and C199/205S gave rise to an active ATPase, demonstrating that the mutated γ-subunits were successfully incorporated into the ATP synthase, although most likely some of the wild-type subunit was also incorporated into the enzyme. Surprisingly, the product from Δ197–205 construct did not integrate into the enzyme implying that, unlike for cyanobacteria, this segment may be important for maintaining a proper conformation and/or stability of the γ-subunit in higher plants.

The mutated γ-subunits are considered to be locked in a "reduced" configuration. Ketcham et al. (1984) created spinach γ-subunit in a "reduced" conformation by incorporation of *N*-ethylmaleimide into cysteine sulfhydryl groups exposed by dithiothreitol treatment of CF_1 in thylakoids. The resulting ATP synthase maintained ATP hydrolysis activity in the dark for several hours compared with less than 30 min in control, similar to the

DTT-reduced ATP synthase in the wild type (Ketcham et al. 1984). Our results on decay of ATP hydrolysis activity in the dark in the serine for cysteine substitution mutants are in agreement with their results. Therefore, we expected that the mutants would not be as efficient as wild type due to the inability to adequately control hydrolysis activity in the dark. Indeed, this was the basis of a mutant screen that we used successfully to isolate chloroplast ATP synthase activation mutants (Gabrys et al. 1994; Gong et al. 2006). Surprisingly, at least under optimal growth conditions, the transformants developed the same and grew at the same rate as wild type at three light levels perhaps indicating there is a restricted accessibility of cellular ATP to the chloroplast ATP synthase in the dark. Thus, it appears that while the presence of the thioredoxin reducible disulfide bond enhances photosynthetic performance (Wu et al. 2007), the physiological role of the reoxidation of the γ-subunit is more subtle.

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